U.S. PATENT APPLICATION

ENTITLED:

PROTEIN BINDING DETERMINATION AND MANIPULATION

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Protein Binding Determination and Manipulation

CROSS REFERENCES TO RELATED APPLICATIONS

This patent application claims priority to provisional patent application No. 60/396,428, filed in the U.S. Patent and Trademark Office on July 17, 2002, the entire contents of which is incorporated herein by reference

FIELD OF THE INVENTION

This invention addresses method for determining protein binding sites, molecules which bind to such sites and molecules which inhibit binding to particular protein binding sites. Also considered are conjoined molecules for enhanced binding to specific protein binding sites.

BACKGROUND OF THE INVENTION

Reference is made to Phage Display of Peptides and Proteins: A Laboratory Manual, Ed. Kay *et al.,* Academic Press, Inc.; "Directed evolution of novel binding proteins," US Pat. No. 5,837,500 (Ladner *et al.*), "Engineering affinity ligands for macromolecules," US Pat. No. 6,326,155 3 (Maclennan *et al.*), "Methods for rapidly identifying small organic molecule ligands for binding to biological target molecules" (Wells *et al.*) US Pat. No. 6,335,155, "Protein tyrosine kinase agonist antibodies," Bennett *et al.* US Pat. No. 6,331,302, and "Monovalent phage display," US Pat. No 5,821,047 (Garrard *et al.*) the teachings of which are incorporated herein by reference. For clarity, the teachings of all patents, journals, texts and publications noted herein are incorporated by reference.

SUMMARY OF THE INVENTION

In one embodiment this comprises a method of obtaining a primary-result peptide having at least one binding domain that binds a predetermined dynamic target material at a non-active site wherein said dynamic target material has at least two conformational energy-minima states comprising:

(a) accessibly-conformationally restraining said dynamic target material in substantially a single conformational energy-minima state

(b) affinity-exposing said accessibly-conformationally restrained single conformational energy-minima dynamic target material to a peptide library comprising inquiry-peptides and identifying peptide which associate with the target with sufficient affinity to withstand washing at least about 4 times in rapid succession with a standard buffer containing physiologically balanced salt solution and a non-ionic detergent (<0.1% v/v) ("peptide hits").

(c) affinity-exposing said accessible conformationally-restrained single conformational energy-minima state dynamic target material to said peptide library wherein said single conformational energy-minima state is substantially a single energy-minima state other than the state of step (a) and identifying peptide-hits; and

(d) selecting at least one peptide-hit that inhibits target function by other-than-competitive inhibition the target material, which peptide-hit being a primary-result peptide.

This invention further includes a method of obtaining a primary-result peptide having at least one binding domain wherein said binding domain is a low affinity binding domain comprising:

(a) preparing a target polypeptide, as a fusion protein having a known target region and an inquiry target region wherein the known target region is linked to the inquiry target region by a flexible linker;

(b) preparing a tandem peptide display library where said tandem peptides comprise

(i) a known peptide element having a binding domain of low affinity as to said known target region said element connected to

(ii) a flexible linker said flexible linker connected to

(iii) an inquiry peptide sequence

(c) affinity exposing said target protein to said peptide library;

(d) identifying tandem peptide-hits ;

(e) identifying said inquiry peptide sequence of said tandem peptide hit as a primary result peptide. In a particular embodiment the method further includes the known target region of (a) comprising an SH3 domain and the known peptide of step (b)(i) comprising a protein-rich SH3 binding domain having an affinity for the known target region with an affinity in the range of 100 micromolar, so as to be of sufficiently low affinity to substantially dissociate from the known target region after washing at most about 4 times in rapid succession with a standard buffer containing physiologically balanced salt solution and a non-ionic detergent (<0.1% v/v). In a particular embodiment, the method further comprises the flexible linker of step (b)(ii) being a short peptide.

In a yet further embodiment the invention comprises a method of obtaining a primary-result peptide useful in inducing formation of activated-like multiprotein complexes bridging two partner polypeptides comprising:

(a) anchoring to a substratum a target polypeptide having a known dimerizable target region, said anchoring being at a location other than said target region and assembling the multiprotein complex, as a ternary complex, by adding a partner target polypeptide and cognate-like accessory polypeptide (such as a hormone) which bridges the two partner polypeptide targets (such as extracellular hormone binding domains of membrane receptors acting as target polypeptides);

(b) exposing said substratum anchored activated-like multiprotein complex to a phage peptide display library and

(c) selecting phage that bind the assembled protein-protein complex with sufficient affinity to withstand washing four times in rapid succession with a standard buffer containing physiologically balanced salt solution and a non-ionic detergent (<0.1% v/v)

(d) selecting from among said complex binding phage a phage that when added to a system containing a substratum anchored target polypeptide and a partner target polypeptide, is capable of inducing the formation of the multiprotein complex such that the two target polypeptide partners become associated in the absence of the accessory polypeptide , said phage bearing a primary result peptide.

Ina further embodiment this invention comprises a method of preparing an enhanced peptide display library comprising preparing a tandem peptide display library having a known target region and an inquiry target region where said tandem peptides comprise

(i) a known peptide element having a binding domain of low affinity as to said known target region said element connected to

(ii) a flexible linker said flexible linker connected to

(iii) an inquiry peptide sequence

(iii) wherein said inquiry peptide sequence is further connected to a bacteriophage structural protein, as well as the library of this method . Particular attention is drawn to an enhanced peptide display library comprising a tandem peptide display library having a known target region and an inquiry target region where said tandem peptides comprise

(i) a known peptide element having a binding domain of low affinity as to said known target region said element connected to

(ii) a flexible linker said flexible linker connected to

(iii) an inquiry peptide sequence

(iii) wherein said inquiry peptide sequence is further connected to a bacteriophage structural protein.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a conceptual drawing of an Erythropoietin receptor (EPOR) with hormone binding domain, amino terminal domain, hormone binding pocket, and carboxyl terminal domain.

Fig. 2 is a conceptual drawing of Erythropoietin (EPO) with a high affinity surface and a low affinity surface.

Fig. 3 is a conceptual drawing of the association of the high affinity surface of an EPO molecule with the hormone binding pocket on an EPOR (an initial event).

Fig. 4 is a conceptual drawing of EPORs anchored on a membrane such that they can only diffuse laterally or rotate in the plane of the membrane. The straight arrow indicates lateral diffusion and the curved arrow indicates rotational diffusion.

Fig. 5 is a conceptual drawing of EPO-EPOR binding. Once the high affinity EPO surface binds to the first EPOR, the low affinity EPO surface is positioned with a narrow two-

dimensional plane. Because the unoccupied EPORs can only diffuse laterally or rotate in that narrow plane, they can easily engage low affinity EPO surface, forming the activated complex.

Fig. 6 is a conceptual drawing of LZHRs. LZHRs are short helical peptides with one face of the helix composed of the amino acid leucine (grey), which has a hydrophobic (water-avoiding) side chain. When two LZHRs are in close proximity the two leucinefaces OzipO together (right), to be shielded from water.

Fig. 7 is a conceptual drawing of the attachment of a short LZHR to EPOR by a flexible linker peptide, the formation of the EPOR*-EPO-EPOR* complex can be effectively achieved in a cell-free environment.

Figures 8 through 17 depict the amino acid side chains to be mutated to the alanine methyl group in the panel of mutants used to identify peptides from a sub-library selected by an initial panning procedure associated with a targeted EOPbp sub-domain.

DETAILED DESCRIPTION OF THE INVENTION

I. Protein topology affixation protocol

One embodiment of the present invention is a protein topology affixation process. The practice of this invention is a process for discovering peptides from combinatorial display libraries that associate with a target enzyme at a non-active site location, and, through such associations, restrict a site specific enzyme from progressing through the changes in conformation necessary for completion of the catalytic cycle peculiar to that enzyme, and in this way inhibit the enzyme's activity by an other-than competitive mechanism (substrate-mimicry).

One use of this process is in drug-development. This process targets the massively-diverse chemical topology of protein surfaces in order to develop drug molecules that are chemically complementary to strategic surface loci with the capacity to restrict the target's

conformational dynamics. In addition this process identifies drug molecules with significantly improved selectivity for individual members of large protein families and develops drug molecules with significantly reduced negative side-effect profiles resulting from improved selectivity.

Conventional target-directed drug discovery has two limited chemical-space data sets available for the design of libraries from which lead compounds are selected, i.e., the structure of the native substrate/ligand and the topology of the target's active-site. The exploitation of both of these data sets has driven the drug-discovery engine of the biotechnology industry.

In a departure from prior design, by the present invention targets are immobilized conformationally prior to ligand determination. In one example of a protocol for enzyme inhibitors (protein tyrosine kinase as example of such enzyme) target immobilization is accomplished as follows:

Targets are immobilized using a c-terminal extension consisting of the peptide sequence (G L N D I F E A Q K I E W H E), unless the c-terminus is integral to target mechanism of action. In the case where the c-terminus of the target is integral to the target's action the peptide sequence can be added to the n-terminus. This peptide sequence is a substrate for *in vitro* biotinylation using a commercially available enzyme, biotin protein ligase, from Avidity, Denver, CO. The biotin-derivatized target is then immobilized on avidin- or streptavidin-coated microtiter plates.

Given the mechanism of target action, two extremes of conformation are identified.

In one extreme the kinase molecule is closed around a non-hydrolysable ATP analog. In the other extreme, the kinase molecule is open with the ATP binding pocket empty.

This process entails affinity isolation of display peptides. In a specific embodiment a bacteriophage peptide display library is applied to the target immobilized in one of the two conformational extremes. Phage that bind to the target are then isolated. The process is repeated with the target held in the other conformational extreme.

Phage characterization is a next step. This includes identification of display peptides specific to one conformational state. Phage clones that associate with the target, held in one of the two conformational extremes are assessed for their ability to bind to the target when it is held in the other conformational extreme. This step identifies those phage clones that bind exclusively to only a single target conformational state. Those single conformational binding phage clones bind to the target at potential function-altering target surface domains. Those phage that bind exclusively to one conformational target state are assessed for their ability to inhibit the activity of the target. Those single conformational binding phage that inhibit the activity of the target are prepared as peptides and assessed. Peptides that perform as the intact phage are advanced. Advanced peptides are assessed for the type of target inhibition, i.e., competitive or other than competitive inhibition, using classical enzyme kinetic analysis.

In one embodiment, peptides that inhibit by other than competitive mechanisms are optimized by affinity maturation to optimize the peptide sequence for binding affinity. The optimized peptides are re-assessed to confirm that target inhibition characteristics are unchanged (or superior). The peptides thus selected are particularly useful in target binding assays used to screen chemical libraries for interaction with the target domains with which the peptide associates. A complimentary use is to determine the chemical-space defined by the peptide's chemistry, employing computational chemistry, in order to design focused combinatorial chemical libraries.

The peptides so identified are also termed protein dynamics modulators (PDMs). PDMs bind to a target, stabilizing one conformational state, preventing progression to other states. PDMs bind to non-active site, functional epitopes on the target's surface (non-competitive / uncompetitive). PDMs modulate target function through restricting the target's structural dynamics. They define the chemical space of the functional epitopes, guiding chemical library design, and are useful in high-throughput screening displacement assays to generate or validate lead compounds.

As noted above PDMs are selected from phage peptide display libraries in a two stage process. First, phage are selected for the ability to bind to immobilized target molecules that are held in one conformational state. Then, phage, identified in stage one, are further selected for the ability to hold the target in the chosen conformational state, preventing the transition to other conformational states. Phage that restrict the target to a single conformational state, and through that restriction inhibit target function, encode for peptides that comprise PDMs.

Examples of proteins usefully restricted in conformational state in the practice of this invention include, the *abl* tyrosine kinase (as well as other kinases), Acetyl CoA carboxylase 2, and other enzymes with particular reference to those of important physiological regulatory significance.

Protocol for enzyme inhibitors
(abl protein tyrosine kinase example)

Target immobilization:

Targets are biotinylated and immobilized on streptavidin-coated microtiter plates. The target sequence is modified on the c-terminus to include the sequence (G L N D I F E A Q K I E W H E), an optimized substrate for biotin protein ligase. The modified target is expressed in a eukaryotic expression system. The c-terminal extension is derivatized with a biotin using biotin

protein ligase (Avidity, Denver, CO). The biotin-derivatized target is then immobilized on streptavidin-coated microtiter plates.

Using knowledge of the mechanism of target action, two extremes of conformation is identified. At one extreme: is the kinase molecule closed around a non-hydrolysable ATP analog. At the other extreme: the kinase molecule open with the ATP binding pocket empty.

Affinity isolation of display peptides:

A bacteriophage peptide display library is applied to a target immobilized in one of the two conformational extremes. Those phage that bind to the target are isolated. Next, the process is repeated with the target held in the other conformational extreme.

Phage characterization:

Identification of display peptides specific to one conformational state:

Phage clones that associate with the target, held in one of the two conformational extremes are assessed for their ability to bind to the target when it is held in the other conformational extreme to identify those phage clones that bind exclusively to only one target conformational state. Those phage clones bind to the target at potential function-altering target surface domains. Those phage that bind exclusively to one conformational target state are assessed for their ability to inhibit the activity of the target. Those phage that inhibit the activity of the target are prepared as peptides. Those peptides that perform as the intact phage are advanced. Advanced peptides are assessed for the type of target inhibition, i.e., competitive or other than competitive inhibition, conveniently, using classical enzyme kinetic analyses. Peptides that inhibit by other than competitive mechanisms are optimized by affinity maturation to optimize the peptide sequence for binding affinity. The optimized peptides are re-assessed to determine if target inhibition characteristics have changed. Those peptides that have retained

their inhibitory characteristics are prepare as conjugates. These conjugates facilitate *in vitro* target detection and are used in target binding assays.

Peptide sequences are analyzed by computational chemistry for the design of focused combinatorial chemical libraries. These libraries are screened for target binding in peptide displacement assays.

II. Low affinity peptide display protocol

Another aspect of this invention uses structural inquiry in discovering and isolating peptides from combinatorial display libraries that associate with a target protein at locations with affinities too low to withstand conventional washing. This technique takes advantage of the multiplicative affinity of conjoined peptides and/or molecules. Low affinity target-interacting peptides from a peptide display library are captured by linking a random display peptide sequence to a constant peptide sequence that has low affinity for an additional protein domain linked to the target protein as a fusion protein by a flexible linker. The affinity for the two (or more) linked peptides is the product of their individual affinities for their respective protein domains. A constant peptide sequence is selected for binding additional protein domain(s) with an affinity low enough to prevent binding to be maintained without an additional binding contribution from the random display peptide. The strategy of employing a binary library identifies peptide sequence families in the random display peptides that otherwise go undetected by conventional panning approaches and the like.

In the process of this aspect of the invention a target is prepared. It is useful to prepare the target protein as a fusion protein such that the target protein is linked by a flexible linker peptide to a protein domain (the bait) known to bind a specific peptide sequence with low

affinity. A specific example target is (abl) fusion protein construct. This construct has an SH3 domain linked to the amino-terminus (or to the carboxyl-terminus) of the target (abl catalytic domain) by a flexible linker peptide (the flexible linker peptide is varied in length to accommodate to varying target sizes).

A library display is then employed. The peptide display library is used so that the constant low-affinity peptide is linked by a short flexible sequence to the random display peptide sequence. In this embodiment one peptide display library consists of two structural peptides linked by a flexible linker peptide sequence. One structural peptide is held constant (*e.g.*, proline-rich SH3 binding peptide sequence). The constant sequence is linked by a short flexible linker peptide with the random peptide display sequence. The constant sequence is chosen for low affinity binding (high micromolar) to the constant domain.

Isolated low affinity peptides are then used as basis for defining or developing higher affinity analogues. In some cases a series of single amino substitutions are made resulting in higher affinity analogues. Other affinity increasing techniques are known in the art. Resulting analogues with increased affinity are useful as peptides that associate with a target enzyme at active or non-active site locations, and, through such associations, restrict a site specific enzyme.

III. Protein-protein interaction inhibitors and method of use.

Yet another one embodiment of this invention includes a process for the discovery of molecules from combinatorial peptide display libraries that block protein-protein interaction, particularly as used in *in vitro* discovery systems. Molecules which block protein-protein interaction by competing for a protein-protein contact surface are useful in defining "surfaces" which induce therapeutic protein-protein interaction.

The present method identifies molecules that block specific protein-protein interactions. Useful points of inquiry are molecules that, (i). are validated as contributing to disease, (ii) are composed of two identified protein targets, (iii). are mediated by structurally defined protein-contact surfaces, and (iv). are difficult to assemble as an *in vitro* assay in a high-throughput screening environment.

The dynamics of EPOR activation by EPO, as shown in Fig. 1, can be reduced to a two step process (EPO itself has a high affinity surface and a low affinity surface as shown in Fig. 2)

- EPO binds to one EPOR (Fig. 3)

1. A second EPOR is recruited to the EPOR-EPO complex creating the EPOR-EPO-EPOR activated complex. The above-noted technique is employed to select PDMs that block the transition from the EPOR-EPO state to the EPOR-EPO-EPOR state and to select PDMs that bind to the EPORs only in the EPOR-EPO-EPOR complex

The PDMs selected in this first example come with inherent advantages that are a direct result of the design of the secondary screening process. Both the PDMs and the EPOR sites to which they bind are chemically and conformationally defined. These comprise target/configuration/binding information useful in the design of the chemical libraries used in drug discovery. As shown in Fig. 5, the activated complex, the PDM binding sites on one EPOR are opposite to and in close proximity to PDM binding sites on the other EPOR. Enhanced binding of PDMs is achieved (i) optimizing initially identified PDMs and then linking two or more PDMs together. Such a linked molecule comprises an activated complex.

In a particular embodiment of this invention one selects PDMs that bind to the EPORs only in the EPOR-EPO-EPOR complex. Note that it is very difficult to form the activated EPOR-EPO-EPOR complex in a cell-free environment. This is because the two EPORs that

come together to form the activated EPOR-EPO-EPOR complex are not restricted to the two-dimensions of the membrane, but are free to diffuse in three dimensions, requiring the second EPOR to be present at extremely high concentrations. EPORs anchored to a membrane are shown in Fig. 4 One approach to overcoming this difficulty is to link an additional structural feature, with a low affinity attraction for itself, to the end of the EPOR (EPOR*).

The affinity for the formation of the EPOR*- EPO-EPOR* complex is the product of the affinities for the two associative events, i.e., the low affinity EPO/EPOR binding is multiplied by the low affinity binding of self-associating linked structure, note Fig. 5.

The leucine-zipper heptad-repeat (LZHR) is useful for the self-associating linked EPOR*- EPO-EPOR* structure. When two LZHRs are in close proximity the two leucine faces "zip" together to be shielded from water as shown in Figs. 6. and 7. The process of selecting phage for candidate PDM identification has two phases,

1. The selection of all phage that bind to the activated EPOR*-EPO-EPOR* complex is a first phase
2. Identification of phage selected in the first round that can induce the formation of an EPOR*-EPOR* complex in the absence of EPO is the second phase.

Note that by attaching a short LZRH to the EPOR by a flexible linker peptide, the formation of the EPOR*-EPO-EPOR* complex can be effectively achieved in a cell-free environment.

A significant embodiment of the invention is the process comprising two phases performed in sequence. In the first phase, one member of a protein-protein interacting pair is immobilized such as on a substrate. Next, display peptides that associate with the target are selected. Selection usefully employs the technique of panning (this approach is compatible with

the anglerfish binary screen technology but other selection techniques are contemplated within this invention). Those display peptides selected in the first phase are then passed through a second phase screen. The second phase screen consists of screening the entities selected in the first-phase panning against a family of target site-directed mutants in which at least one and in some embodiments all charged amino acid residues residing on the inter-protein contact surface have been changed to the amino acid alanine. First-phase selectants that associate with the inter-protein contact surface are identified by their ability to associate with the wild type (non-mutated) target and all but a subset of mutant target molecules. The subset of mutants to which the first-phase selectant fails to bind identifies the target inter-protein contact surface loci to which the selectant binds.

More specifically in Phase One a target protein is prepared with an amino or carboxyl terminal extension useful for immobilizing the target *in vitro* so that target function is largely unperturbed and substantially the full target surface area is accessible to the media. Panning technology collects members of a combinatorial peptide display library that specifically associate with the target.

The target (*e,g.*, erythropoietin receptor extracellular hormone binding domain (ERHBD)) is generated with amino-terminal peptide extension (G L N D I F E A Q K I E W H E). The lysine residue (K) is biotinylated enzymatically (ERHBD*) and the construct is immobilized on avidin-coated plastic plates. Proper target folding is established by determining epo binding. A combinatorial peptide display library, preadsorbed on avidin coated plates saturated with biotin, is then applied to the immobilized ERHBD*, and those elements of the library associating with the ERHBD* are collected. The collected elements are "phase-one selectants".

Immobilization technology is exemplary of the approach. Other techniques that capture the target without altering its surface structure are adequate.

In Phase Two a family of target protein constructs in which charged amino acid residues present on the protein-protein contact surface are individually mutated to the amino acid alanine. The wild type (non-mutated) and the alanine mutant constructs are then immobilized as an array in microtiter plates and the Phase One selectants are screened for binding to the array. Those Phase One selectants that bind to the protein-protein contact surface are identified by their binding to the wild type and all but a subset of the mutant constructs. Those mutants that exclude the Phase one selectants identify the surface locus to which the selectants bind.

In the ERHBD-epo-ERHBD complex, the carboxyl-terminal fibronectin type III (FNIII) domains of the two ERHBD are positioned opposite each other. The charged amino acid residues located within the protein-protein contact region are R130, D133, E134, R141, R171, E173, E176, R178, E180, and R187 (R=arginine (+), D=aspartic acid (-), and E=glutamic acid (-)). Ten individual ERHBD* mutants are constructed in which each of the listed charged amino acid residues are mutated to alanine (this is a classical strategy used to assess the role of specific amino acid side chains in biochemical processes). The wild type ERHBD* construct and each of the ERHBD* alanine-mutants are then immobilized as an array in avidin-coated microtiter plates, i.e., wild type in column 1, R130A in column 2, D133A in column 3, E134 in column 4, R141 in column 5, R171 in column 6, E173 in column 7, E176 in column 8, R178 in column9, E180 in column 10, R187 in column 11, and wild-type in column 12. The individual Phase One selectants are then dispensed into individual rows and their ability to bind to the immobilized array of ERHBD* constructs are assessed. Those Phase One selectants that bind equally to all of the ERHBD* constructs in the row bind to ERHBD regions that are outside of the protein-

protein contact region. Those Phase One selectants that bind to the wild type and all but one or a subset of the alanine mutants are identified as binding to a locus within the protein-protein contact region. Furthermore, the specific alanine mutant(s) that exclude the selectant define the surface location to which the selectant binds.

By this embodiment, the selectants define a "chemical space" for the design of chemical libraries to search for drug leads that perform as the selectant. The selectants are particularly useful as chemical tools in high-throughput screening assays to identify chemical entities that compete with the selectant for the same target surface locus, identifying the chemical entity as a drug lead.

IV. Enhanced Combinatorial Peptide Display Library

A further embodiment of this invention provides enhanced combinatorial peptide-display libraries in which the displayed peptide is ribosome-associated, and the RNA encoding the peptide is retained as a ribosome-associated RNA. This allows for collection of positive clones by panning, with the encoding RNA recoverable as well for cloning, and sequencing.

In this embodiment of peptide display technology, bacteriophage biology is not obligatory. The instant approach exploits a feature of the prokaryote translation system, i.e., the ability of an RNA molecule lacking a termination codon to lock a ribosome into a quasi-stable "ternary complex" consisting of the peptide-ribosome-mRNA. This complex can be captured by a variety of methods including panning protocols and the encoding RNA can be recovered and cloned, providing a connection between associating peptide and the mRNA sequence encoding it. This approach increases the potential chemical diversity of the display library and accommodates novel scaffolds not readily adaptable to phage display. An additional advantage is the elimination of any requirement for the peptide fold to be permissive of phage viability.

When the prokaryote -translation apparatus is translating an mRNA that abruptly terminates without a stop codon the mRNA/ribosome/nascent polypeptide chain complex becomes locked into a quasi-stable complex we will refer to as a Frozen Translation Unit (FTU). *In vivo*, this complex is conveniently recovered by a process that employs two bacterial components that work together, small protein B (spB) and transfer-messenger RNA (tmRNA). The recovery process is initiated by tmRNA and spB binding to the vacant tRNA binding site on the FTU. Once the spB/tmRNA binds to the ribosome in the vacant "A" tRNA binding site the nascent polypeptide chain is transferred to tmRNA. The synthesis of the protein molecule is completed using a quasi-mRNA sequence that is part of the tmRNA structure. To capture FTUs from an *in vitro* translation system spB and tmRNA are removed from the *in vitro* translation system.

The mRNA family encoding for the combinatorial peptide array is generated by any convenient methods of *in vitro* mutagenesis. Useful vectors and templates have an RNA pol start transcription site upstream of the multi cloning site. A polypeptide template that has been cloned into the multicloning site usefully has a flexible carboxyl terminus capable of presenting the display peptide at a distance from the ribosome, what ever constant domains are included, and a flexible linkage between the constant domain and the variegated peptide (if necessary), with the variegated occupying the amino terminus of the displayed polypeptide

V. Modulation of Protein-Protein Interactions

The process of this invention yet further includes isolation and identification of reagents that block specific protein-protein interactions (PPI_{br}). In particular such protein-protein interactions occur as the result of one protein molecule bridging two or more other protein molecules. In some embodiments of this process having known atomic coordinates for the

formed multi-protein complex is advantageous. The goals of the process, however, are also achieved with a less rigorous structural foreknowledge. The PPI_{br} discovered by this process are usefully assembled into structures. By way of example, with epo there are 2 identical EPOR molecules that approach close enough such that their intracellular domains interact sufficiently to allow signal propagation. Thus, a structure is determined by the process of this invention that associates with the face of the c-terminal FNIII domain that serves as a steric block to the approach of the second EPOR. In "assembly," two of these structures are joined with their FNIII domain contact surfaces facing in opposite direction. Such a molecule binds to one EPOR and is positioned to "compel" a second EPOR molecule to associate into a bi-receptor complex that positions the two intracellular domains close enough together to facilitate signal propagation. of the multi-protein complex in the absence of the bridging protein molecule. Without being bound by any particular theory its is believed that the receptors are conveniently viewed as "transducing elements", as they have structures in both the extracellular and intracellular compartments, and they communicate (or transduce) the signal, represented as a constituent in the extracellular space (the hormone epo) to the intracellular environment (the intracellular domains that propagate the signal). One utility of this approach is generation of orally available therapeutic antagonist and agonist molecules. Particular utility for such molecules in cancer treatment and hormone replacement therapy. In hormone replacement-therapy it is therapeutic to establish hormonal sufficiency in a state where the hormone is being under produced. In such cases treatment with an agonist is useful. For example a peptide that activates the receptor in the same manner as the hormone does (treating diabetes with insulin, kidney failure with EPO, post-menopause with estrogen, castration with testosterone, etc). For cancer chemotherapy, in instances where there is an excessive hormonal stimulus, such as from a hormonal

overproduction or expression of a receptor fueling cell growth it is desirable to block the action with an antagonist (IGF-I in some prostate and breast cancer, EGF in some solid tumors, testosterone in prostate cancer, growth hormone in acromegaly).

Example:
Selective PPI_{br}

A PPI_{br} [protein-protein interaction blocking reagent] is designed to block the formation of the activated complex consisting of two erythropoietin receptors bridged by one protein molecule (here erythropoietin), but not, in this example, block the interaction of one erythropoietin receptor with an erythropoietin molecule. This PPI_{br}, blocks the accretion of the second erythropoietin receptor to the pre-formed erythropoietin receptor-erythropoietin complex.

Information, materials, and methods useful in PPI_{br} preparation include:

- The extracellular domain of the human erythropoietin receptor
    - Modifications described in Syed et al (1998) Nature 395:515 for expression in eukaryote expression systems (CHO or Pichia pastoris) is described in Table 1 (the product will be referred to as EPObp) (For the quantities required for the described exercise, the CHO, 293 EBNA, or other cell culture systems will be adequate or are adjusted in a manner known by one of ordinary skill in the art.).
    - An additional alteration to the EPObp is added at the amino terminus to facilitate immobilization of the target EPObp in streptavidin coated microplates. By "alteration" it is meant that: any amino- or carboxyl-terminal change which facilitates immobilization or affixation is usefully (and optionally) included. Alternatively no alteration need be made. Reference is made to optional use of an

antibody to the amino-terminal FNIII domain that doesn't interfere with EPO binding.

- The sequence (G L N D I F E A Q K I E W H E) is added to the amino-terminus of the EPObp. Without being bound by any particular theory it is believed to allow the *in vitro* enzymatic biotinylation of the EPObp in accordance with the recommendations of Avidity (Denver, CO).

- A panel of EPObp charge-to-alanine mutants is generated. In one embodiment EPObp charge-to-alanine mutants comprise amino acids on the carboxyl-terminal FNIII domain, with charged side chains that project into the space between the two opposing EPORs in the ternary complex (EPOR-EPO-EPOR). (R=arginine, D=aspartic acid, E=glutamic acid, A=alanine) (see Table 2)
  - R130A
  - D133A
  - E134A
  - R141A
  - R171A
  - E173A
  - E176A
  - R178A
  - E180A
  - R187A

- Human erythropoietin (EPO) (unlabled and labeled with ^{125}I) will be used to establish proper folding of the EPObp constructs by assessing EPO binding isotherms in classical competition assays.

- Bacteriophage peptide display libraries (libraries)
- Conjugated antibodies directed against non-varigated bacteriophage coat proteins for use in detecting bound bacteriophage using a microplate reader.

Process description:

- Initial panning step
  - Pre-adsorb the library with the immobilization matrix minus the target, i.e., streptavidin coated wells without b-EPObp to remove library components with affinity for binding the matrix, in this example.
  - Adsorb the pre-adsorbed library with immobilized b-EPObp
    - Sequential harvesting
      - Remove the supernatant and retain as devoid of binders (0)
      - Wash once and retain as containing the weakest binders (1)
      - Wash a second time and retain as containing weak binders (2)
      - Wash a third time and retain as containing poor binders (3)
      - Wash a forth time and retain as containing modest binders (4)
      - Wash a fifth time and retain as containing moderate binders (5)
      - Elute the remaining material and retain as containing strong binders (6)
- Assessment of strong binders

- Clone the strong binders using accepted practices, and assess 96 clones for insert size and insert sequence.
- Choose those clones containing inserts with non-identical sequences for primary selection
- Prepare microplates in which columns 1 and 12 contain b-EPObp, and in which columns 2-11 contain the individual charge to alanine mutants, described above, i.e., 2=R130A, 3=D133A, 4=E134A, 5=R141A, 6=R171A, 7=E173A, 8=E176A, 9=R178A, 10=E180A, and 11=R187A.
- Each clone to be evaluated is incubated with an entire row of microplates prepared as described in the preceding step, i.e., the native b-EPObps in wells 1 and 12, and each of the charge to alanine mutants in wells 2-11.
    - Following incubation each well is washed.
    - Each well is than incubated with the anti-bacteriophage conjugated antibody.
    - Un-bound antibody is removed by washing.
    - Each well is incubated with the chromogenic substrate and the amount of bound bacteriophage is estimated by the color intensity assessed by the mictoplate reader.
- Assessment of strong binders
    - Those bacteriophage that bind equally to each well of the row are declared to bind to b-EPObp surfaces distinct from those defined by the locations of the charge to alanine mutations , and probably bind to the EPO binding site.

- Those bacteriophage that bind to wells 1 and 12, as well as most of the other wells, but not all of the other wells, are declared to bind to a region of the b-EPObp defined by the specific charge to alanine mutants to which the bacteriophage fails to bind. For example, if the bacteriophage binds to all wells except wells 8 and 9, then the bacteriophage likely associated with the EPOR near E178 and R178.

- All of the bacteriophage that are identified by the above screening protocol as associating with the circumscribed protein surface are optimized for affinity by affinity maturation, synthesized as peptides and reassessed for binding. Those peptides that behave as the phage guide the design of chemical libraries, using computational chemistry. The chemical libraries are then screened for target binding by displacement of the conjugates, cognate peptide to discover drug leads.

Table 1

EPOR swiss prot accession # p19235

Key	From	To	Length	Description
SIGNAL	1	24	24	
CHAIN	25	508	484	ERYTHROPOIETIN RECEPTOR.
DOMAIN	25	250	226	EXTRACELLULAR (*POTENTIAL*).
TRANSMEM	251	273	23	*POTENTIAL*.
DOMAIN	274	508	235	CYTOPLASMIC (*POTENTIAL*).
DOMAIN	148	213	66	FIBRONECTIN TYPE-III.
DISULFID	52	62		
DISULFID	91	107		
CARBOHYD	76	76		N-LINKED (GLCNAC...) (*POTENTIAL*)

```
      10        20        30        40        50        60
      |         |         |         |         |         |
MDHLGASLWP QVGSLCLLLA GAAWAPPPNL PDPKFESKAA LLAARGPEEL LCFTERLEDL

      70        80        90       100       110       120
      |         |         |         |         |         |
VCFWEEAASA GVGPGNYSFS YQLEDEPWKL CRLHQAPTAR GAVRFWCSLP TADTSSFVPL

     130       140       150       160       170       180
      |         |         |         |         |         |
ELRVTAASGA PRYHRVIHIN EVVLLDAPVG LVARLADESG HVVLRWLPPP ETPMTSHIRY

     190       200       210       220       230       240
      |         |         |         |         |         |
EVDVSAGNGA GSVQRVEILE GRTECVLSNL RGRTRYTFAV RARMAEPSFG GFWSAWSEPV

     250       260       270       280       290       300
      |         |         |         |         |         |
SLLTPSDLDP LILTLSLILV VILVLLTVLA LLSHRRALKQ KIWPGIPSPE SEFEGLFTTH

     310       320       330       340       350       360
      |         |         |         |         |         |
KGNFQLWLYQ NDGCLWWSPC TPFTEDPPAS LEVLSERCWG TMQAVEPGTD DEGPLLEPVG

     370       380       390       400       410       420
      |         |         |         |         |         |
SEHAQDTYLV LDKWLLPRNP PSEDLPGPGG SVDIVAMDEG SEASSCSSAL ASKPSPEGAS

     430       440       450       460       470       480
      |         |         |         |         |         |
AASFEYTILD PSSQLLRPWT LCPELPPTPP HLKYLYLVVS DSGISTDYSS GDSQGAQGGL

     490       500
```

SDGPYSNPYE NSLIPAAEPL PPSYVACS
EPOR construct as described in Syed et al 1998 Nature 395:515
A25 redefined as aa#1
specific mutations shown in red: N52Q, N164Q, and A211E
The ala, shown in orange was replaced by arg-glu-phe (REF)

10 20 30 40 50 60
~~MDHLGASLWP QVGSLCLLLA GAAW~~APPPNL PDPKFESKAA LLAARGPEEL LCFTERLEDL

70 80 90 100 110 120
VCFWEEAASA GVGPGQYSFS YQLEDEPWKL CRLHQAPTAR GAVRFWCSLP TADTSSFVPL

130 140 150 160 170 180
ELRVTAASGA PRYHRVIHIN EVVLLDAPVG LVARLADESG HVVLRWLPPP ETPMTSHIRY

190 200 210 220 230 240
EVDVSAGQGA GSVQRVEILE GRTECVLSNL RGRTRYTFAV RARMAEPSFG GFWSEWSEPV

250 260 270 280 290 300
SLLTPSDLDP ~~LILTLSLILV VILVLLTVLA LLSHRRALKQ KIWPGIPSPE SEFEGLFTTH~~

310 320 330 340 350 360
~~KGNFQLWLYQ NDGCLWWSPC TPFTEDPPAS LEVLSERCWG TMQAVEPGTD DEGPLLEPVG~~

370 380 390 400 410 420
~~SEHAQDTYLV LDKWLLPRNP PSEDLPGPGG SVDIVAMDEG SEASSCSSAL ASKPSPEGAS~~

430 440 450 460 470 480
~~AASFEYTILD PSSQLLRPWT LCPELPPTPP HLKYLYLVVS DSGISTDYSS GDSQGAQGGL~~

490 500
~~SDGPYSNPYE NSLIPAAEPL PPSYVACS~~

Table 2

Charge to alanine EPObp mutants//those amino acids depicted in red will be individually changed to alanine

10 20 30 40 50 60

~~MDHLGASLWP QVGSLCLLLA GAAW~~APPPNL PDPKFESKAA LLAARGPEEL LCFTERLEDL

70 80 90 100 110 120

VCFWEEAASA GVGPGQYSFS YQLEDEPWKL CRLHQAPTAR GAVRFWCSLP TADTSSFVPL

130 140 150 160 170 180

ELRVTAASGA PRYHRVIHIN EVVLLDAPVG LVARLADESG HVVLRWLPPP ETPMTSHIRY

190 200 210 220 230 240

EVDVSAGQGA GSVQRVEILE GRTECVLSNL RGRTRYTFAV RARMAEPSFG GFWSEWSEPV

250 260 270 280 290 300

SLLTPSDLDP ~~LILTLSLILV VILVLLTVLA LLSHRRALKQ KIWPGIPSPE SEFEGLFTTH~~

310 320 330 340 350 360

~~KGNFQLWLYQ NDGCLWWSPC TPFTEDPPAS LEVLSERCWG TMQAVEPGTD DEGPLLEPVG~~

370 380 390 400 410 420

~~SEHAQDTYLV LDKWLLPRNP PSEDLPGPGG SVDIVAMDEG SEASSGSSAL ASKPSPEGAS~~

430 440 450 460 470 480

~~AASFEYTILD PSSQLLRPWT LCPELPPTPP HLKYLYLVVS DSGISTDYSS GDSQGAQGGL~~

490 500

~~SDGPYSNPYE NSLIPAAEPL PPSYVACS~~

Figures 8 through 17 depict the amino acid side chains to be mutated to the alanine methyl group in the panel of mutants used to identify peptides from a sub-library selected by an initial panning procedure associated with a targeted EOPbp sub-domain. Numbers on the figures

are counted from the amino terminus. The orientation of the EPObp seen in R130 (Fig. 8), D133 (Fig. 9), E134 (Fig. 10), and R141 (Fig. 11), R171 (Fig. 12), E172 (Fig.13), E176 (Fig. 14), R178 (Fig. 15), E180 (Fig 16) and R187 (Fig. 17) are of the EPObp in rightward rotational views.